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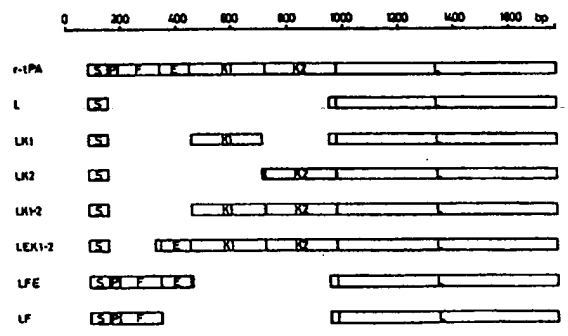
(54) Tissue-type plasminogen activator mutants; recombinant genetic information coding therefor and process for preparing said mutants; their use and pharmaceutical compositions.

(57) The invention relates to tissue-type plasminogen activator (t-PA) mutants having improved binding characteristics towards fibrin. The binding of t-PA to fibrin is due to the kringle K2 domain and, to a lesser extent, to the finger domain F. The claimed mutants either comprise at least one K2 or at least one F domain and lack one or more other parts of the t-PA molecule, or comprise at least two K2 or F domains, or comprise at least one modified K2 or F domain having improved binding characteristics towards fibrin. The invention also relates to a process for preparing said mutants by genetic engineering and to the recombinant genetic information used therein. Further, the invention relates to the use of said mutants in therapy or imaging and to pharmaceutical compositions suitable therefor.

FIG.1



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Tissue-type plasminogen activator mutants; recombinant genetic information coding therefor and process for preparing said mutants; their use and pharmaceutical compositions.

The present invention relates to tissue-type plasminogen activator (t-PA) mutants having improved binding characteristics towards fibrin compared to naturally occurring t-PA. This invention also relates to a process for preparing said mutants by genetic engineering technology and to the recombinant genetic information used therein. Finally, the invention also relates to the use of said mutants in therapy or imaging and to pharmaceutical compositions which are suitable for said purposes.

Tissue-type plasminogen activator (t-PA) is a serine protease which converts the zymogen plasminogen into plasmin, a serine protease which degrades the fibrin network (1). The activity of t-PA is substantially accelerated in the presence of fibrin, a property which has focussed the attention on t-PA as a potential therapeutic anti-thrombogenic agent - (2-4). The molecular mechanism of the stimulatory influence of fibrin is based on the notion that both the substrate plasminogen and the enzyme t-PA are bound to and aligned on the fibrin matrix, facilitating a localized generation of plasmin.

t-PA is synthesized and secreted by vascular endothelial cells as a single-chain polypeptide (5). This molecule is converted by plasmin or trypsin into a two-chain polypeptide connected by a disulfide bond. The heavy (H) chain of t-PA (M_r 39 000) is located at the amino-terminus, whereas the light (L) chain (M,33 000) is at the carboxy-terminal end (6). Based on the amino-acid sequence homology of t-PA with both the trypsin family of serine proteases, prothrombin, epidermal growth factor and fibronectin, a model for the secondary structure of t-PA has been proposed (6,7). In this model, different structural domains have been assembled to create a composite mosaic polypeptide. The L-chain of t-PA was proposed to harbor the serine protease moiety, responsible for plasminogen activator activity. Recently, we found evidence which demonstrates that in mammalian cells separately expressed t-PA L-chain cDNA is indeed solely capable to convert plasminogen into plasmin and this activity is not accelerated by fibrin. The H-chain of t-PA contains considerable amino-acid homology with several plasma proteins. Apart from a typical signal peptide (S) and a prosequence (P), similar to those of serum albumin (8,9), one can distinguish a 'finger' domain (F) resembling the regions on fibronectin involved in fibrin binding (10,11) and a structure partially homologous to both human and mouse epidermal growth factor (E) (12,13). Furthermore, two 'kringle' structures are proposed to be situated on the carboxy-terminal part of the H-chain, highly homologous to structural and functional domains on plasminogen, shown to be involved in fibrin binding of plasminogen (14).

The elucidation of the chromosomal structure of the t-PA gene and the alignment with the complete amino-acid sequence revealed in intriguing observation, namely that exons or sets of exons coincide with the proposed structural domains - (6,15,16). Those studies and others have led to the hypothesis that mosaic proteins, such as t-PA and other plasma proteins, are composed of different functional 'modules' as a result of 'exon shuffling', an evolutionary rearrangement event (17,18).

Herein, we provide evidence that structural domains in the t-PA protein, encoded by separate exons or sets of exons, harbor autonomous functions.

Materials and methods

Materials

Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase and E-scherichia coli DNA polymerase I (Klenow fragment) were purchased from New England Biolabs. Mung Bean nuclease was from P.L. Biochemicals Inc., Tunicamycin from Calbiochem, Iscove's modified medium from Flow Laboratories and all reagents required for the plasminogen activator assay were from Kabi Vitrum.

General methods

Plasmid DNA was isolated by a modification of the alkaline–lysis procedure (19), followed by CsCl-equilibrium centrifugation. Enzyme reactions were carried out using standard conditions (20). Nucleotic sequence determinations were performed using the method of Maxam and Gilbert (21).

Construction of expression plasmids

Plasmid ptPA8FL consists of vector pBR322 and full-length t-PA cDNA (2,540 bp) inserted by G-C tailing into the PstI site of the vector. Human t-PA cDNA was constructed using Bowes Melanoma polyA+RNA. A fragment (2,087 bp) of the full length t-PA cDNA, extending from bp 78 to 2,165 - (numbering of the t-PA cDNA is according to Pen-

nica et al. (6)) and containing the entire coding sequence, was subcloned between the HincII and BamHI sites of the pUC9 polylinker, resulting in plasmid pUCtPA. A HindIII-SacI fragment of pUCtPA was inserted together with a SacI-Bg1II fragment of ptPA8FL into the HindIII and Bg1II sites of PSV2 (22) to yield pSV2tPA. This plasmid contains the origin of replication and the β-lactamase gene of pBR322, the SV40 early promotor, the t-PA-coding sequence and SV40 splice and polyadenylation sequences. All t-PA deletion-mutant expression plasmids were derived from pSV2tPA by constructing deletions in the coding sequence using restriction sites adjacent to the junctions of the hypothetical domains. To preserve the translation reading frame, in some of the constructions restriction fragment termini were altered using DNA-modifying enzymes (details are given in the legend to Fig.1). DNA-sequence determinations were performed to verify a correct in-phase ligation of all new cDNA junctions in each construct. We made a deposit on February 6, 1986 with the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands, of an E. coli K12DH1 containing the plasmid pSV$_2$/t-PA.

Tissue culture and transfection

Mouse Ltk$^-$cells were maintained in Iscove's modified minimal medium, containing penicillin, streptomycin and 10% fetal calf serum. Transfection was carried out essentially as described (23). After transfection, the cells were incubated in serum-free Iscove's medium, containing penicillin, streptomycin and 0.3 mg/ml acid-treated bovine serum albumin. In some of the experiments, tunicamycin (1 µg/ml) was added. Five days after transfection, cell media regularly contained 1 to 5 µg rt-PA or rt-PA mutant proteins per 8x10^6 cells.

Gelatin-plasminogen gelelectrophoresis

Electrophoresis on gelatin gels, containing 13 µg/ml copolymerized plasminogen, was performed essentially as described (24). Ten per cent polyacrylamide gels, containing 0.1% sodium dodecylsulfate, were incubated after electrophoresis in 2.5% Triton-X 100 for 2 h to remove sodium dodecylsulfate. Subsequently, the gels were incubated for 6 h in 0.1 M glycin-NaOH (pH 8.3). Gelatin degradation, due to plasminogen activation in the gel, was visualized by contrastaining using 0.1% amidoblack.

Plasminogen activator assay

Plasminogen activator activity was determined using an indirect spectrophotometric assay (27). This assay measures the amidolytic activity of plasmin, which is generated by the plasminogen activator-catalyzed conversion of plasminogen and is based on the hydrolysis of a bond in the chromogenic substrate D-val-leu-lys pNitro anilide (S2251). Assays were performed at 37°C in 250 µl 0.1 M Tris HCl (pH 7.5), containing 0.13 µM plasminogen, 0.1% Tween-80, 120 µg/ml CNBr-digested fibrinogen (when indicated) and 0.30 mM S2251. The absorbance at 405 nM was followed for 6h.

Immunoradiometric assay for t-PA L-chain antigen

Goat-anti-mouse IgG coupled to Sepharose beads was incubated with limiting amounts of the murine monoclonal anti-human t-PA L-chain IgG - (ESP2) (25) and with a ^{125}I-radiolabeled t-PA tracer in phosphate-buffered saline, containing 1% bovine serum albumin, 0.1% Tween-80 and 10 mM EDTA. The incubation was performed end over end for 18 h at room temperature in stoppered polystyrene tubes. Separation of bound and free radiolabeled tracer was carried out by centrifugation for 2 min at 3000xg. The Sepharose beads were washed 4 times with 2 ml 0.9 M NaCl, 0.1% Tween-80, 10 mM EDTA and bound radioactivity was determined in a gamma-counter. Unlabeled Bowes Melanoma t-PA was used as a competitor for the binding of ^{125}I-t-PA. Approximately 100 ng t-PA, in a final volume of 0.5 ml, reduced the binding of the tracer till 50% of the maximum value. Conditioned media, containing rt-PA or rt-PA mutant protein, were also used as competitor for radiolabeled t-PA to bind to the monoclonal ESP2 IgG. A comparison between the percentage of competition of the conditioned media and serial dilutions of Bowes Melanoma t-PA allows an estimation of the amount of L-chain antigen present.

Results

Construction of t-PA deletion-mutant expression plasmids

To study the biological properties of the separate hypothetical domains of t-PA, we have constructed a series of t-PA cDNA expression plasmids which systematically lack the coding sequence of one ore more of the domains on the H-chain. For that purpose, we have employed our cloned full-length t-PA cDNA, composed of a 95-bp

5'-untranslated region, a 1,686-bp-coding region and a 759-bp 3'-untranslated region. Restriction-enzyme analysis and partial nucleotide-sequence determinations revealed identity with the DNA sequence reported by Pennica et al.(6). Sections of full-length t-PA cDNA were inserted into a 'shuttle vector' (pSV2), consisting of parts of plasmid pBR322 and the eukaryotic virus SV40. In these constructs, t-PA cDNA is preceded by the SV40 early promoter and linked at its 3' end to splice and polyadenylation signals. All constructs have two features in common. First, they harbor the coding sequence for the t-PA signal peptide (nucleotides 85 till at least 156) to ensure secretion of the - (mutant) polypeptides. Second, all constructs contain the entire coding region for the t-PA L-chain - (nucleotides 953 till 1,771). The latter domain is responsible for the plasminogen activator activity, thereby allowing a convenient assay of the biological activity. Furthermore, the involvement of the different domains of the H-chain on the stimulation of the plasminogen activator activity of t-PA by fibrin can be readily assessed. Essential junctions of the constructs were sequenced to verify the continuity of the translation reading frame. A - schematic representation of the H-chain t-PA cDNA-deletion mutants is shown in Fig. 1.

Characterization of the expression products

Mouse Ltk⁻cells appeared to be suitable hosts for transient expression of rt-PA, programmed by t-PA cDNA-harboring plasmids, because these cells can be efficiently transfected and do neither contain nor secrete plasminogen activator(s). The different expression products, present in the serum-free media 5 days after transfection, were analyzed by gelatin-plasminogen gel electrophoresis (not shown). This technique localizes plasminogen activator activity in SDS-polyacrylamide gels. The method depends on the principle that plasminogen and gelatin, when incorporated into the polyacrylamide matrix at the time of casting, are retained during electrophoresis. In-situ plasmin formation and consequently degradation of gelatin can be visualized by negative staining. Clearly, Melanoma t-PA and rt-PA display similar mobilities in this gel system. The rt-PA-deletion mutants were also secreted, apparently due to routing directed by the signal peptide. The mobility of the deletion-mutant proteins corresponds with the expected values. Obviously, all mutant proteins exhibit a basal plasminogen activator activity in accord with our previous observations that the L-chain suffices for this activity. To demonstrate that the heterogeneity of the products results from differential glycosylation, tunicamycin, an inhibitor of N-glycosylation, was added to the cell media during the expression of the products. The results (not shown) were that under these conditions all the proteins display single bands on the gelatin gel, indicating that they are expressed as unique polypeptides. Our data and those of others (26) show that the carbohydrate moieties of t-PA are not involved in its biological activity. We consider the expression products to be valuable tools in studying the biological properties of the as yet hypothetical domains of the t-PA molecule.

Plasminogen activation by the mutant proteins; the influence of fibrinogen fragments

To investigate the effect of fibrin on the plasminogen activator activity of the deletion-mutant proteins, we used an amidolytic assay, relying on the chromogenic substrate S2251. We determined the activity of rt-PA and rt-PA mutant proteins in the presence and absence of cyanogen-bromide-digested fibrinogen, a digest known to mimic the potentiating the effect of fibrin (27). The data are given in Fig. 2. In the absence of fibrinogen fragments, rt-PA only has a basal activity, but upon adding fibrinogen fragments, the plasminogen activator activity is greatly accelerated. The mutant proteins L and LK1 display only a basal activity, and no significant stimulation of the plasminogen activator activity by fibrinogen fragments was detected. In contrast, the plasminogen activator activity of the mutant proteins LEK 1-2, LK1-2 and LK2 was stimulated by fibrinogen fragments to the same magnitude as rt-PA. The plasminogen activator activity of the mutant proteins LFE and LF (not shown) were also stimulated by fibrinogen fragments, but to a lesser extent than the K2-domain-containing mutant proteins. These results indicate that the K2 domain of the t-PA H-chain and, to a lesser extent, the finger (F) region mediate the stimulatory effect of fibrin. Finally, 'kringle' K1 and the EGF region apparently do not contribute to the stimulatory mechanism. It is assumed that the molecular basis for the stimulation by fibrin is that both plasminogen and t-PA are bound by the fibrin polymer, hereby aligning t-PA with its substrate - (3,28). Therefore, we examined the fibrin-binding properties of the t-PA deletion-mutant proteins.

Fibrin binding

Fibrin matrices were formed in the presence of either rt-PA or rt-PA deletion-mutant proteins and subsequently pelleted (29). Plasminogen activator activity of supernatants and of solubilized pellets were analyzed on gelatin-plasminogen gels (not

shown). In the case of rt-PA and L, the total amount used (input), the non-binding and the binding fractions were investigated. Most of the rt-PA-input fraction was bound to the fibrin matrix, whereas no significant binding of the L-chain protein was detected. The input and the fraction bound to fibrin of the other proteins were also investigated. LK1 does not exhibit significant binding to fibrin. The K2-domain-containing mutants LEK1-2, LK1-2 and LK2 bound to fibrin to about the same extent, although not as efficient as the binding of rt-PA. The LFE and LF mutants also bound to fibrin, but to a lesser extent than the K2-domain-containing mutant proteins. Hence, the stimulatory effect of fibrinogen fragments on the plasminogen activator activity, mediated by kringle K2 and to a lesser extent by the F region, correlates well with the fibrin-binding characteristics of these domains. Apparently, the acceleration of the plasminogen activator activity of t-PA is intimately linked to binding to the fibrin matrix.

Discussion

The elucidation of the t-PA gene structure by Ny et al. (15) revealed that separated exons or separate sets of exons encode structural domains, which were postulated on the basis of homology with other plasma proteins. To test whether these autonomous structural domains indeed have autonomous functions, we have expressed a series of rt-PA deletion-mutant proteins lacking one or more of the structural domains and studied the remaining biological properties. Earlier studies from this laboratory showed that separately expressed L-chain molecules harbor the plasminogen activator activity, however, this activity could not be stimulated by fibrin. In those studies, the L-chain molecules were not secreted by the tissue-culture cells that were used for expression of L-chain cDNA. In this paper, we have been able to show that efficient secretion of the expression products occurs, provided the signal peptide is encoded at the 5' end of the cDNA of different deletion mutants. This result indicates that the t-PA signal peptide has an autonomous function.

The finger (F) domain exhibits a fibrin-binding function and also mediates in part the stimulatory effect of 'fibrin' on plasminogen activator activity of t-PA.

Clearly, the K2 domain has an autonomous function in the binding of fibrin as well as in mediating stimulation of the plasminogen activator activity of t-PA by fibrin. Surprisingly, the kringle K1 domain does not seem to be involved in the fibrin-binding property of t-PA. Although, the amino-acid sequence of kringle K1 and K2 are highly homologous and the secondary structure might be similar (6,7), apparently subtle differences may specify fibrin affinity. Such differences might explain the lack of fibrin affinity of urokinase (u-PA), in spite of the presence of a kringle structure on this molecule.

We propose that the fibrin-binding characteristics of t-PA are mediated both by the finger domain (F) and the K2 domain, the latter contributing most to the binding. Additional evidence for the involvement of the kringle K2 domain to fibrin binding was gained by the observation that a murine monoclonal anti-human t-PA antibody (ESP5) (25), which was found to inhibit in part fibrin binding of t-PA, specifically bound to LK2, but not to the L-chain product (results not shown). The fact that this conformation-dependent monoclonal antibody reacted equally well with rt-PA as with the K2-domain-containing mutant proteins indicates that the K2 domain retains its native conformation in the deletion-mutant proteins.

Mosaic proteins (e.g.t-PA) have been proposed to be evolved by 'exon shuffling', an evolutionary process linking different functional structures. By showing that structural domains in t-PA, encoded by separate exons or sets of exons, are autonomous functional domains, our study supports the concept of exon shuffling as a mechanism to create new genes. In our view, the approach explored here to investigate the structure-function relationships of the t-PA molecule with these and other deletion mutants may elucidate which domains are involved in the complex formation of t-PA with plasminogen activator inhibitor(s) (30,31) and in the binding of t-PA to the receptor(s) in the liver (32,33), responsible for the rapid clearance of t-PA from the bloodstream.

Summarizing, we employed transfected mouse LtK$^-$ cells for transient expression of recombinant human tissue-type plasminogen activator (rt-PA) or of rt-PA deletion-mutant proteins, encoded by SV40-pBR322-derived t-PA cDNA plasmids. The t-PA cDNA-deletion mutants have two features in common, i.e. cDNA programming the signal peptide and the coding region for the L-chain. Consequently, rt-PA mutant proteins are efficiently secreted and display plasminogen activator activity. The N-terminal H-chain, being an array of structural domains homologous to other plasma proteins ('finger', 'epidermal growth factor', 'kringles'), was mutated using restriction endonucleases to delete one or more structural domains. The stimulatory effect of fibrinogen fragments on the plasminogen activator activity of t-PA was demonstrated to be mediated by the kringle K2 domain and to a lesser extent by the finger region, but not by the kringle K1 and the EGF domains. These data correlate well with the fibrin-binding properties of the rt-PA

deletion-mutant proteins, indicating that the stimulation of the activity by fibrinogen fragments is based on aligning the substrate plasminogen and t-PA on the fibrin matrix. Our results also support the evolutionary concept of 'exon shuffling', arranging structural domains which constitute autonomous functions of the protein.

Our work as described above provides the understanding and tools necessary for the designing and actual production of t-PA mutants having improved binding characteristics towards fibrin compared to naturally occurring t-PA forms and possibly to t-PA mutants having more desirable properties in the complex formation with plasminogen activator inhibitor(s) and/or the binding to the receptor(s) in the liver.

A direct result of our work is the development of pharmaceutical compositions comprising t-PA mutants having improved binding characteristics towards fibrin, which can be beneficial in therapy for conditions of fibrin clot arrested blood circulation, such as, for example, venous thrombosis, arterial thrombosis, myocardial infarction and lung emboly. Of course the t-PA mutants used for therapy purposes must at least comprise the L-chain which harbors the plasminogen activator activity. This requirement does not exist in a second kind of use of novel t-PA mutants having improved binding characteristics towards fibrin, which is their use in imaging for localizing fibrin clots, such as, for example, a thrombus. Then, however, the mutants should comprise a detectable label, such as, for example, a radio-isotope.

Pharmaceutical compositions containing t-PA are known (4) and men in the art will therefore have no problems whatsoever in formulating pharmaceutical compositions containing t-PA mutants as claimed herein.

Although different administration routes can be employed, normally intravenous, intraarterial or intracoronarial administration will be preferred to obtain quickest results. For these administration routes, the pharmaceutical compositions will normally comprise an aqueous solution of the t-PA mutant, optionally further containing conventional additives, such as, for example, additives which assist in solving the t-PA mutant, as is well-known for aqueous solutions containing t-PA itself.

Legends to Figures

Fig.1 -Schematic representation of t-PA cDNA present in the rt-PA and rt-PA deletion-mutant expression vectors. rt-PA is coded for by the entire coding cDNA. Deletion-mutant cDNAs were constructed by fusing t-PA restriction-fragment termini that were in some cases enzymatically modified to preserve the translation reading frame. L [deletion of base pair 158 till (not including) 953 (Δ 158-953)] was constructed by fusing the EcoRII(153)-terminus [filled in by E.coli DNA polymerase-I large fragment (Klenow)] to the ScaI(950)-terminus. LK2 - (Δ 156-711); fusion of the BstNI(153)-terminus - (Klenow) to the DdeI(710)-terminus (Klenow). LK1-2 (Δ158-458); fusion of the EcoRII(153)-terminus - (Klenow) to the HaeIII(456)-terminus. LK1 (Δ 158-458/Δ713-953); as LK1-2 with one extra deletion made by fusion of the DdeI(710)-terminus [partially filled in by Klenow (TTP, dGTP) followed by a Mung Bean nuclease treatment] to the ScaI(950)-terminus. LEK1-2 (Δ 158-326); fusion of the EcoRII-(153)-terminus (Klenow) to the DraIII(319)-terminus (T4-DNA polymerase, adding dATP only, followed by a Mung Bean nuclease treatment). LFE (Δ 455-953); fusion of the BstNI(452)-terminus (Klenow) to the ScaI(950)-terminus. LF (Δ 332-953) has been constructed in two steps. a)LK1-2 was digested with NarI (517) and ScaI(950) and treated with Klenow. A BbvI fragment (252-341) was isolated from pSV2/t-PA DNA digested with BbvI. This fragment was incubated with T4-DNA polymerase, dATP, dCTP, TTP and subsequently with Mung Beam nuclease. This modified fragment was inserted into LK1-2, prepared as described above. b) The resulting plasmid was digested with DraIII - (at 319 and 1365; lacking 332-953). The DraIII fragment, containing the deletion, was employed to substitute for the 'intact' DraIII fragment (319-1365) of pSV2/t-PA to yield LF.
DNA sequencing showed that either T4-DNA polymerase or Mung Bean nuclease has 'nibbled' at the BbvI ends. S, signal peptide; P, propeptide; F, finger domain; E, EGF domain; K1 and K2, kringle domains, and L, light chain.

Fig.2 -The influence of 'fibrin' (fibrinogen fragments) on the plasminogen activation by rt-PA, u-PA, L, LK1, LK2, LK1-2, LEK1-2 and LFE proteins as determined with an amidolytic assay using the chromogenic substrate S2251 (27). 2 mU u-PA or 20 µl serum-free medium containing about 0.15 pmoles (determined by the immunoradiometric assay) of the different expression products were used. The absorbance at 405 nm was followed for at least 4 h and conditioned serum-free medium of Ltk⁻cells, that were transfected with promotorless ptPA8FL DNA, was used as a control. O, + fibrinogen fragments; Δ, -fibrinogen fragments.

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Claims

1. Tissue-type plasminogen activator (t-PA) mutants, comprising at least one kringle domain K2 or at least one finger domain (F), or both, including modified forms having equivalent or improved binding strength, binding selectivity, or both, towards fibrin, and either lacking at least part of the natural molecule or containing at least two K2 and/or F domains, having improved binding strength, binding selectivity, or both, towards fibrin.

2. t-PA mutants of claim 1, comprising the signal peptide (S), at least one K2 domain, and the light chain (L), and lacking at least part of the propeptide (P), the epidermal growth factor domain (E), the kringle domain K1, and the F domain.

3. t-PA mutants of claim 1, comprising S, at least one F domain, and L, and lacking at least part of P, E, K1 and K2.

4. t-PA mutants of claim 1, comprising S, at least one K2 domain, at least one F domain, and L, and lacking at least part of P, E and K1.

5. t-PA mutants of claim 1, comprising S, P, L and at least one K2 domain, or at least one F domain, or both and lacking at least part of E and K1.

6. t-PA mutants of claim 1, comprising S, P, L, E and at least one K2 domain, or at least one F domain, or both, and lacking at least part of K1.

7. t-PA mutants of claim 1, comprising S, P, L, K1 and at least one K2 domain, or at least one F domain, or both, and lacking at least part of E.

8. t-PA mutants of claim 1, comprising S, L, K1 or E or both, and at least one K2 domain, or at least one F domain, or both, and lacking at least part of P.

9. t-PA mutants of claim 1, comprising S, P, E, F, K1 and L and two or more K2 domains.

10. t-PA mutants of claim 1, comprising S, P, E, K1, K2 and L and two or more F domains.

11. t-PA mutants of claim 1, comprising at least one modified K2 domain, F domain, or both, having improved binding strength, binding selectivity, or both, towards fibrin.

12. Recombinant genetic information in the form of RNA, single strand DNA or double strand DNA coding for t-PA mutants of claim 1.

13. Recombinant cloning and/or expression vector, such as, for example, a plasmide, comprising a suitable cloning and/or expression vehicle having inserted therein the recombinant genetic information of claim 12.

14. A process for preparing t-PA mutants of claim 1, wherein a suitable host microorganism or cell culture transformed by a recombinant expression vector of claim 13 is grown and the t-PA mutants produced therein are isolated.

15. Microorganism being transformed by a recombinant vector of claim 13 and being capable of cloning and/or expression of the recombinant genetic information of claim 12.

16. Cell culture being transformed by a recombinant vector of claim 13 and being capable of cloning and/or expression of the recombinant genetic information of claim 12.

17. Use of L-containing t-PA mutants of claim 1 in therapy for venous thrombosis, arterial thrombosis, myocardial infarction, lung emboly, and other conditions of fibrin clot arrested blood circulation.

18. Use of t-PA mutants of claim 1, provided with a detectable label such as, for example, a radio-isotope, in imaging for localizing a fibrin clot, such as, for example, a thrombus.

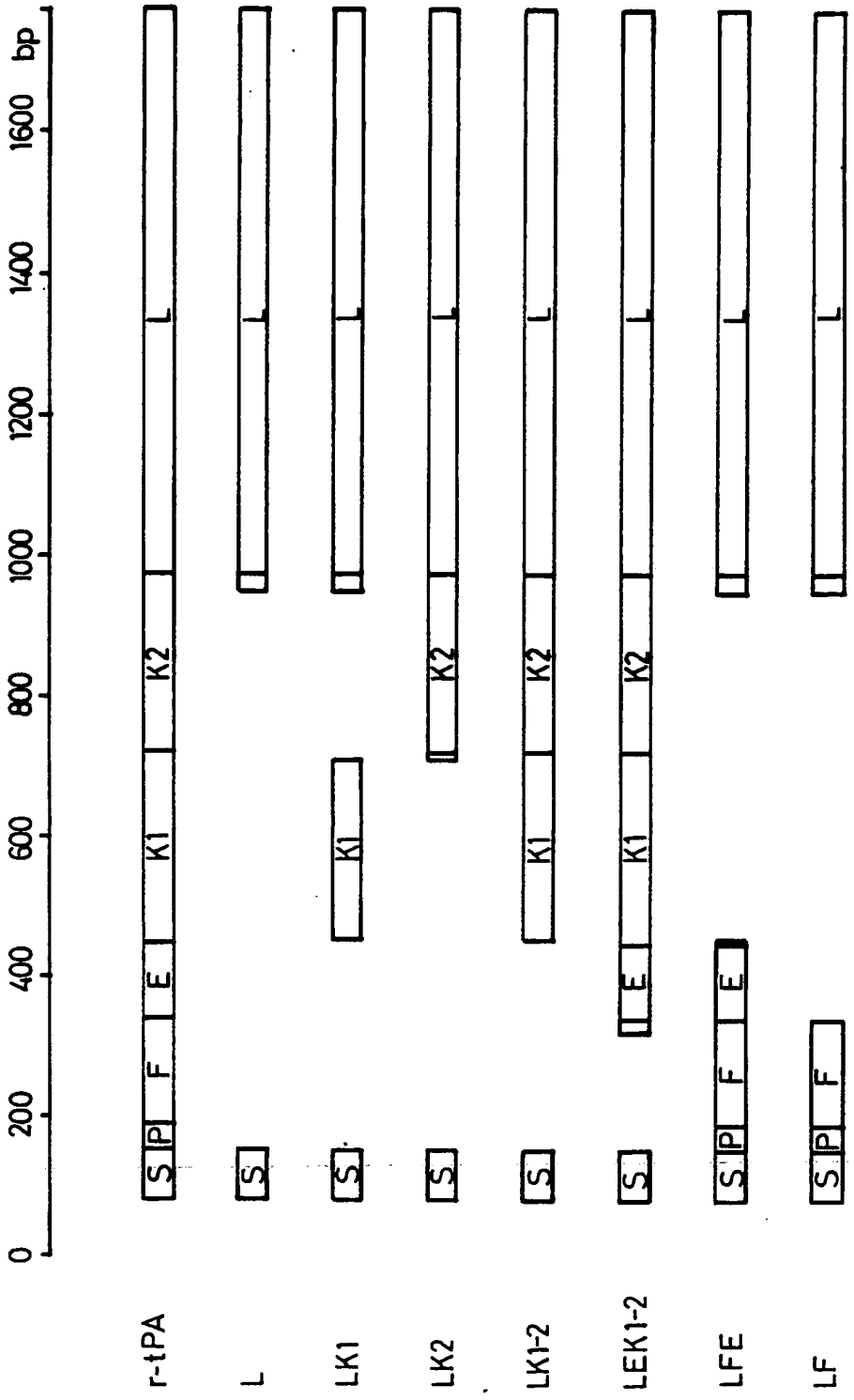
19. Pharmaceutical composition for use in therapy for venous thrombosis, arterial thrombosis, myocardial infarction, lung emboly, and other conditions of fibrin clot arrested blood circulation, comprising an L-containing t-PA mutant of claim 1 and at least one pharmacologically acceptable carrier, diluent or vehicle.

20. Pharmaceutical composition of claim 19, comprising an aqueous solution of a L-containing t-PA mutant of claim 1, which is suitable for intravenous, intraarterial, or intracoronarial administration.

21. Pharmaceutical composition for use in imaging for localizing a fibrin clot, such as, for example, a thrombus, comprising a suitably labeled t-PA mutant of claim 1 and at least one pharmacologically acceptable carrier, diluent or vehicle.

22. Pharmaceutical composition of claim 21, comprising an aqueous solution of a suitably labeled t-PA mutant of claim 1, which is suitable for intravenous, intraarterial or intracoronarial administration.

FIG.1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bp
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L
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LF
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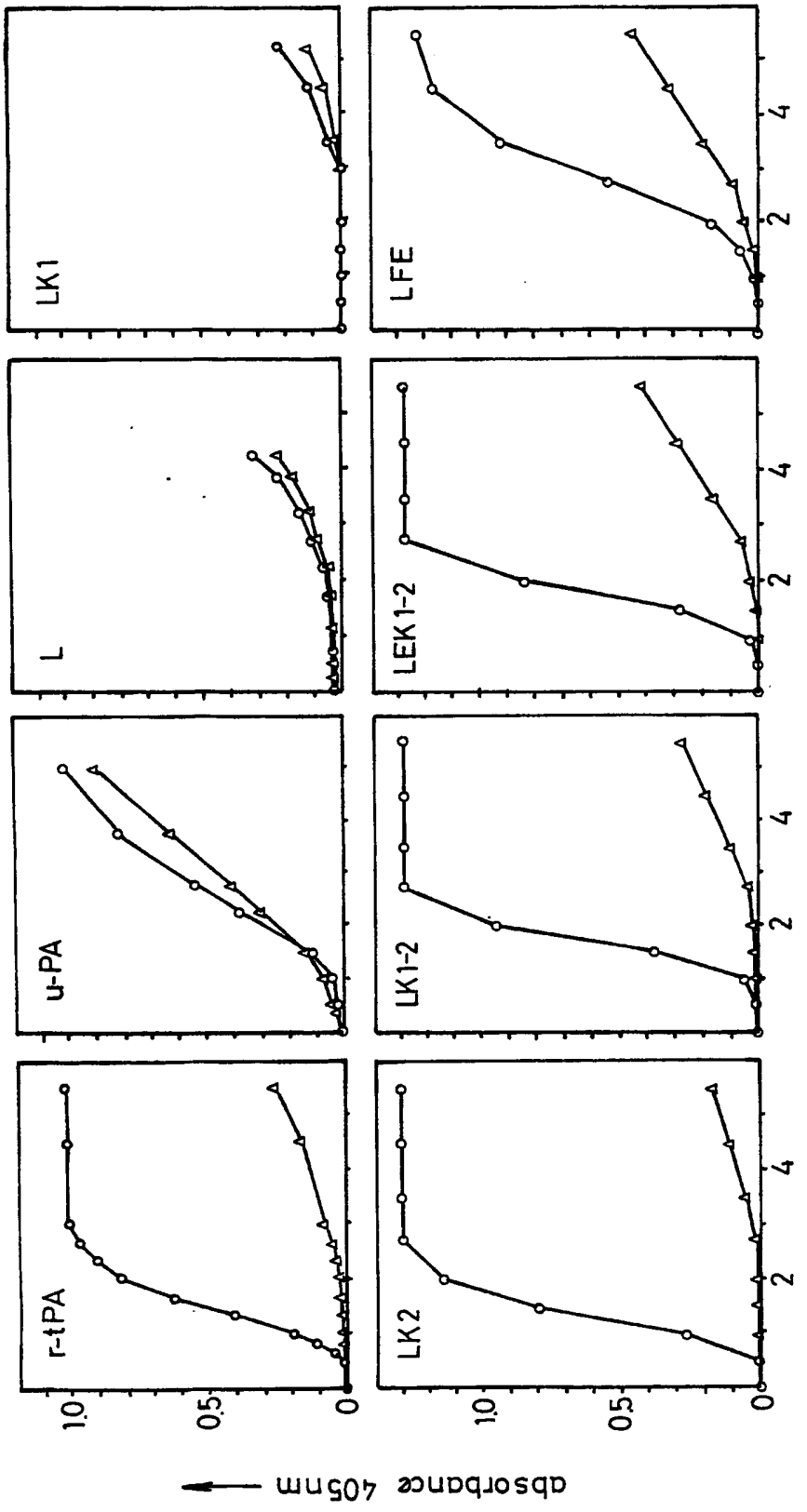
P
F
E
K1
K2
L

FIG. 2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LK1
LK2
LK1-2
LEK1-2
LFE
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0
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4
absorbance 405nm
time: hours

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application number
EP 86 20 0223

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
D,A	EP - A - 0 093 619 (GENENTECH) * claims * --	1	C 12 N 9/64 C 12 N 15/00 C 12 N 1/20 A 61 K 37/54
A	GENE, volume 33, no. 3, 1985, pages 279-284 Elsevier Science Publishers AMSTERDAM (NL) M.J. BROWNE et al.: "Isolation of a human tissue-type plasminogen-activator genomic DNA clone and its expression in mouse L cells" * whole document * --------	1	

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

C 12 N
A 61 K

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.

Claims searched completely:

Claims searched incompletely:

Claims not searched: 17,18

Reason for the limitation of the search:

Method for treatment of the human or animal body by surgery or therapy (see art. 52(4) of the European Patent Convention)

Place of search	Date of completion of the search	Examiner
THE HAGUE	17-10-1986	DELANGHE

CATEGORY OF CITED DOCUMENTS

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